EDGEWOOD CHEMICAL BIOLOGICAL CENTER
U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1178

# METABOLOMIC ANALYSIS OF THE SECRETOME OF HUMAN EMBRYONIC STEM CELLS FOLLOWING METHYL PARATHION AND METHYL PARAOXON EXPOSURE

## PHASE II: METABOLITE DOWNSELECTION FOR STRUCTURAL CONFIRMATION

**Janna S. Madren-Whalley**
**Jennifer W. Sekowski**

**RESEARCH AND TECHNOLOGY DIRECTORATE**

**Paul R. West**
**Elizabeth L. R. Donley**
**Robert E. Burrier**

**STEMINA BIOMARKER DISCOVERY, INC.**
**Madison, WI 53719-1256**

**December 2013**



U.S. ARMY RDECOM

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| XX-12-2013 | Final | Oct 2011 – Jan 2012 |

**4. TITLE AND SUBTITLE:**
Metabolomic Analysis of the Secretome of Human Embryonic Stem Cells Following Methyl Parathion and Methyl Paraoxon Exposure, Phase II: Metabolite Downselection for Structural Confirmation

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S):**
Madren-Whalley, Janna S.; Sekowski, Jennifer W. (ECBC); West, Paul R.; Donley, Elizabeth L. R.; Burrier, Robert E. (Stemina)

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Director, ECBC, ATTN: RDCB-DRB, APG, MD 21010-5424
Stemina Biomarker Discovery, Inc., 504 South Rosa Road, Suite 150, Madison, WI 53719-1256

**8. PERFORMING ORGANIZATION REPORT NUMBER**
ECBC-TR-1178

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Edgewood Biological Chemical Center (ECBC), In-House Laboratory Independent Research (ILIR) Program, Aberdeen Proving Ground, MD 21010-5424

**10. SPONSOR/MONITOR'S ACRONYM(S)**
ECBC ILIR

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution is unlimited.

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT:**

This technical report is the second of three reports from the U.S. Army Edgewood Chemical Biological Center (ECBC) In-House Laboratory Independent Research funded project, Molecular Toxicology of toxic industrial chemicals (TICs) in Human Embryonic Stem Cells (hESC). One of the goals of the study was to identify metabolites from the secretome of hESC exposed to the TICs methyl parathion and methyl paraoxon. A nontargeted liquid chromatography followed by mass spectrometry (LC-MS) approach was used in the Phase I work (Sekowski, J.W., et al. *Metabolomic Analysis of the Secretome of WA09 Human Embryonic Stem Cells following Methyl Parathion and Paraoxon Exposure, Phase I: Initial Nontargeted LC-MS;* ECBC-TR-1177; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2013). In the Phase I work, hundreds of human metabolites and multiple metabolic pathways altered as a result of exposure to both chemicals were putatively identified. The purpose of this Phase II study was to downselect putatively identified metabolites for structural confirmation analysis by liquid chromatography followed by tandem mass spectrometry (LC-MS-MS). This report describes the subset of mass features that were downselected based on the following criteria: (1) impact on the arginine-proline metabolism pathway, (2) involvement with reactive oxygen species, and (3) good quality mass features suitable for structure confirmation by LC-MS-MS.

**15. SUBJECT TERMS**

| | | |
|---|---|---|
| Human embryonic stem cells (hESC) | Methyl parathion (MP) | LC-MS |
| Metabolomics | Methyl paraoxon (MPO) | Reactive oxygen species (ROS) |

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | Renu B. Rastogi |
| U | U | U | UU | 32 | **19b. TELEPHONE NUMBER** *(include area code)* (410) 436-7545 |

Blank

# PREFACE

The work described in this report was authorized under a U.S. Army Edgewood Chemical Biological Center (ECBC) In-House Laboratory Independent Research (ILIR) Program during the 2012 fiscal year. The work was started in October 2011 and completed in January 2012.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.


## Acknowledgments

The authors acknowledge the following individuals for their hard work and assistance with the execution of this technical program:

- Way Fountain (ECBC) and Laura Borland (Booz Allen Hamilton, Aberdeen, MD) for their support of this project under the ECBC ILIR program.
- Kelley Betts (ECBC) for her technical assistance in the preparation of this manuscript.

CONTENTS



FIGURES



TABLES

Blank

# METABOLOMIC ANALYSIS OF THE SECRETOME OF HUMAN EMBRYONIC STEM CELLS FOLLOWING METHYL PARATHION AND METHYL PARAOXON EXPOSURE, PHASE II: METABOLITE DOWNSELECTION FOR STRUCTURAL CONFIRMATION

## 1. INTRODUCTION

This technical report is the second of three reports from the U.S. Army Edgewood Chemical Biological Center (ECBC) In-House Laboratory Independent Research (ILIR) "Molecular Toxicology of TICs in Human Embryonic Stem Cells". The overarching goal of the study was to provide a better understanding of the basic biological and toxicological mechanisms in human embryonic stem cells (hESC) exposed to an organophosphate toxic industrial chemical (TIC). This work has broad implications toward many U.S. Army goals from developing better toxicant screening tools, including organ-on-a-chip type applications, to medical applications, including regenerative medicine applications and stem cell based therapies. It also supports U.S. Army Training and Doctrine Command (TRADOC) Pamphlet (Pam) 525-66 (March 08) *Maneuver Sustainment*, Force Operating Capabilities (FOC)-09-07 – "Casualty Prevention", which states the importance of protecting the warfighter from TICs and toxic industrial materials (TIM)s in general.

Chemicals that have certain known effects in adults can have dramatically different toxic effects during embryonic and prenatal development. For example, the successful adult anti-epileptic drug, valproate, has a dramatic toxic effect on embryonic development, leading to neural tube deficits, autism, and cognitive dysfunction. In their 2007 study, Cezar et al. used liquid chromatography followed by mass spectrometry (LC-MS) to examine the metabolites in spent medium from pluripotent hESC exposed to valproate and found a blockage of the serotonin production pathway. Therefore, an important part of any complete toxicological evaluation must include examination of the compound's effect on human embryonic development. The use of hESC to explore human embryonic molecular toxicological endpoints is a promising development in the field of toxicology. Since pluripotent hESC contain the ability to differentiate into any somatic cell in the body, they provide a unique window into the influence of toxicants on the entire early human development process.

To address the aim of this study, we used nontargeted LC-MS based metabolomic analysis of the secretome of hESC exposed to methyl parathion (MP) and methyl paraoxon (MPO), the active metabolite of MP. In the course of this work, we also confirmed that hESC are not able to convert parathion to paraoxon, thus in order to understand the effect of both the parent compound and its metabolite, the testing of both compounds was necessary. This nontargeted LC-MS approach was able to identify hundreds of human metabolites and multiple metabolic pathways that were altered as a result of exposure to both chemicals that would be present in vivo.

The purpose of this Phase II study was to refine parts of the analysis and down-select metabolites of interest from the previous initial Phase I work described in Sekowski et al. (2013). That technical report describes the results from initial LC-MS based untargeted metabolomic analysis that was performed on hESC exposed to MP or MPO. The goal of this

work was to identify metabolites secreted from those cells that were altered by exposure to MP or MPO. Based on the initial results, further literature-based investigation revealed that several specific metabolic processes, including the arginine and proline metabolism pathway and the involvement of reactive oxygen species (ROS) should be included in subsequent analysis. This report delineates a set of statistically significant mass features that were putatively annotated and determined to be involved with these and other key metabolic processes. Of these, those mass features best suited for structural confirmation by liquid chromatography followed by tandem mass spectrometry (LC-MS-MS) were chosen. That work is reported in the Phase III Technical Report (Madren-Whalley et al., 2013).

## 2. EXPERIMENTAL OVERVIEW

Statistically significant mass features from the Phase I study were re-analyzed by hand to determine the quality of the features for LC-MS-MS analysis. In order to determine the features which had the best potential for structural confirmation, the following criteria were used: (1) sufficient abundance ($1x10^3$), (2) fold change (comparing dosed to controls), and (3) peak shape. After this review and putative annotation of these features, the raw LC-MS data files were re-analyzed using the "Find by Formula" routine in the Agilent (Santa Clara, CA) MassHunter software. This semi-automated analysis determines if the mass spectra for a feature (i.e., the specific molecular formula for the annotation) is a reasonable match with the formula. This is much faster than human manual data interpretation. The algorithm takes into account several factors, including parts per million (ppm) mass error, adducts and isotopic peak abundances, isotope spacing, and mass per charge (m/z) values. It outputs a score, where 100 is perfect, for each feature. Any features with a score of less than 70 were removed. Other factors also considered were potential for ionization for a given polarity [e.g., a quaternary cation such as choline will not be detected in negative ion electrospray ionization (ESI)] and previously confirmed compound retention time matches. All of these factors were taken into account resulting in a list of good quality features for consideration for inclusion in the Table. All sample preparation and LC-MS methods are described in detail in the Phase I Technical Report (Sekowski et al., 2013).

## 3. RESULTS

The table shows the 20 putative metabolites which passed criteria for structural confirmation analysis by LC-MS-MS. This table includes the pathways revealed in the Phase I work described in ECBC TR-1177, as well those involved with ROS and pathways with known biomarkers of developmental toxicity such as arginine and proline metabolism, revealed in this Phase II work. All of these putative metabolites have passed criteria for good likelihood of structural confirmation whether they were detected via the automated data analysis pipeline or by manual inspection.

Table. Putative Metabolites Passing Criteria for Potential LC-MS-MS Confirmation

| Metabolic Pathway | No. of Features in Pathway | Putative Metabolite | Study Phase |
|---|---|---|---|
| Arginine and proline metabolism | 8 | Asymmetric dimethylarginine (ADMA) | II |
| | | Ornithine | II |
| | | L-proline | II |
| | | 2-oxo-5-aminovalerate | II |
| | | Trans-4-hydroxy-L-proline | II |
| | | L-glutamic-gamma-semialdehyde | II |
| | | 4-acetamidobutanoic acid | II |
| | | cis-4-hydroxy-D-proline | II |
| D-arginine and D-ornithine metabolism | 4 | Ornithine | II |
| | | 5-amino-2-oxopentanoic acid | II |
| | | 2-amino-4-oxo-pentanoic acid | II |
| | | (2R,4S)-2,4-diaminopentanoate | II |
| Gly, Ser, and Thr metabolism | 3 | L-cystathionine | II |
| | | Choline | II |
| | | 5-aminolevulinic acid | II |
| Val, Leu, and Ile degradation | 2 | Alpha-ketoisovaleric acid | II |
| | | Valine | II |
| Val, Leu, and Ile biosynthesis | 2 | Alpha-ketoisovaleric acid | II |
| | | Valine | II |
| Lys degradation | 2 | Pipecolic acid | II |
| | | 2-keto-6-aminocaproate | II |
| Pyruvate metabolism | 1 | Malic acid | I |
| Purine metabolism | 1 | Guanine | I |
| Propanoate metabolism | 1 | Valine | II |
| Pantothenate and CoA biosynthesis | 1 | Alpha-ketoisovaleric acid | I |
| Glyoxylate/dicarboxylate metabolism | 1 | Malic acid | I |
| Glycerophospholipid metabolism | 1 | Choline | II |
| Glutathione metabolism | 1 | Ornithine | II |
| Cys and Met metabolism | 1 | L-cystathionine | I |
| Citrate cycle (TCA cycle) | 1 | Malic acid | I |

ADMA, asymmetric dimethylarginine
CoA, coenzyme A
Cys, cysteine
Gly, glycine
Ile, isoleucine
Leu, leucine
Lys, lysine
Met, methionine
Ser, serine
TCA, trichloroacetic
Thr, threonine
Val, valine

All of the pathways in the table have been mapped or remapped to reflect the 20 putative metabolites which pass the criteria for LC-MS-MS analysis described above. The location of the metabolites in the pathways are illustrated below in Figures 1–15. The arginine and proline metabolism pathway (Figure 1) had the most perturbation with the most metabolites that passed the criteria set for best prospect of successful LC-MS-MS based structural confirmation.

## 4. KEGG Pathways

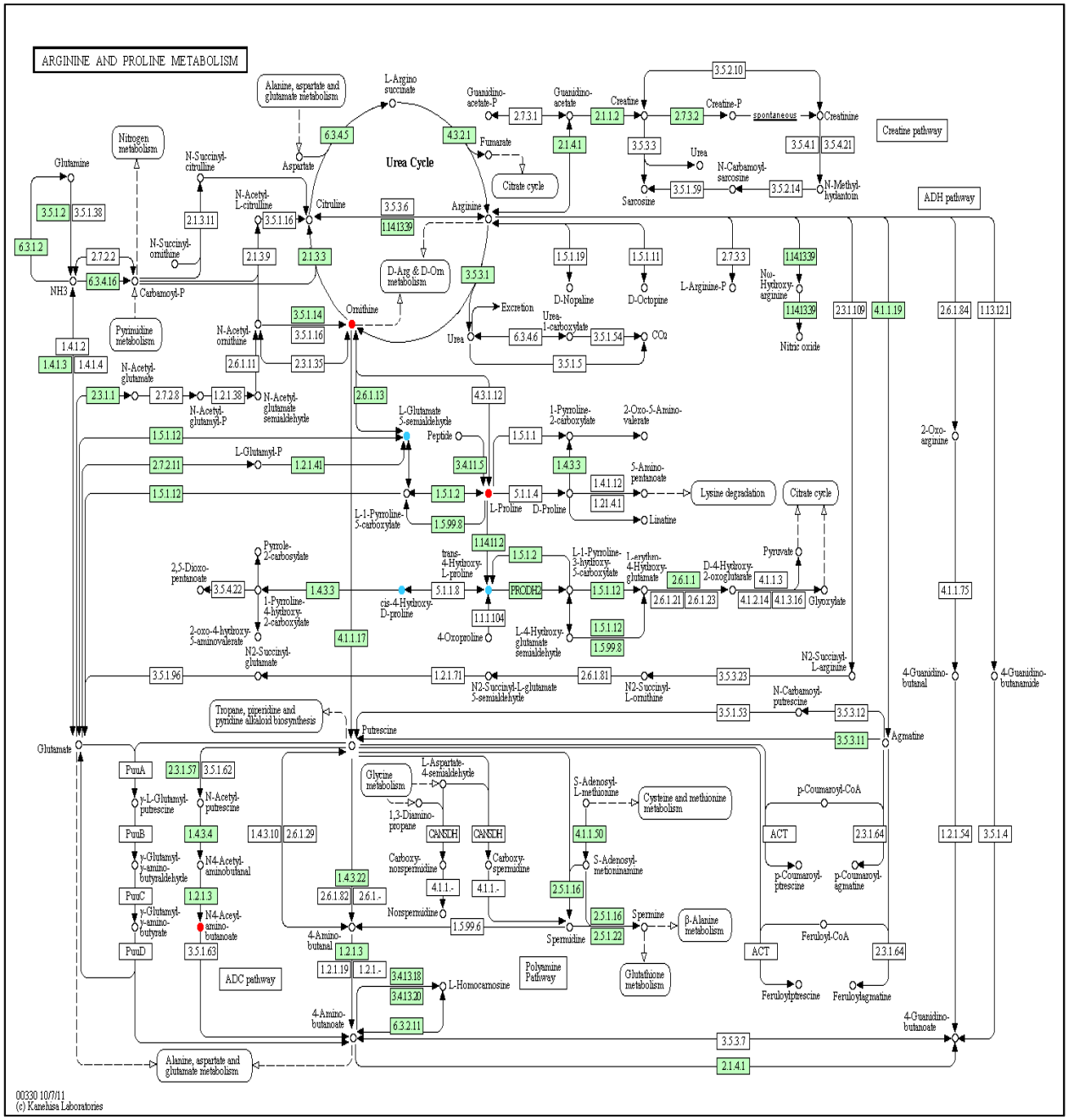


Figure 1. Krypto Encyclopedia at Genes and Genomes (KEGG) pathway diagram of argenine and proline metabolism.

KEGG pathway diagram of the putatively annotated metabolites ornithine, L-proline, 2-oxo-5-aminovalerate, 4-acetamidobutanoic acid (red dots) and L-glutamic-gamma semialdehyde, trans-4-hydroxy-L-proline and cis-4-hydroxy-D-proline (blue dots indicating isomers) and ADMA, an inhibitor of nitric oxide synthase (NOS), in the arginine and proline metabolism pathway that were determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

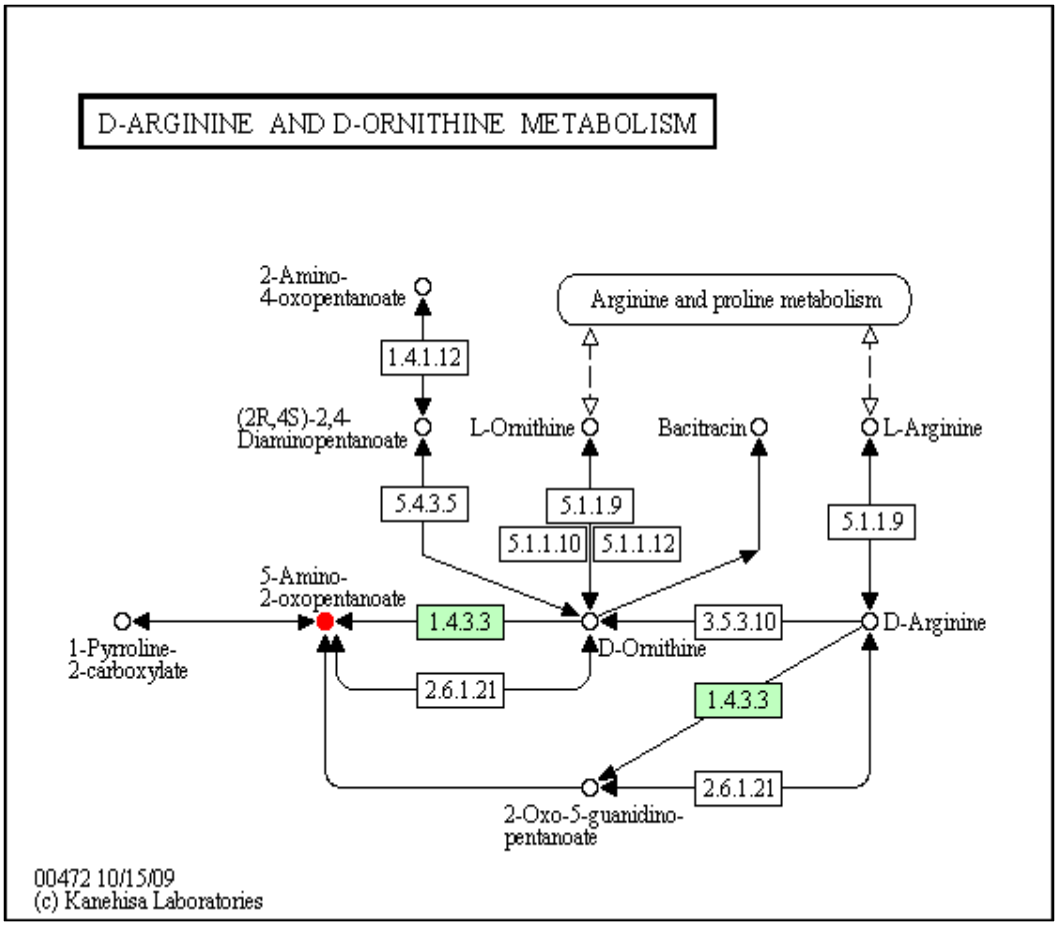


Figure 2. KEGG pathway diagram of D-argenine and D-ornithine metablolism.

KEGG pathway diagram of the putatively annotated metabolite 5-amino-2-oxopentanoate (red dot) in the D-arginine and D-ornithine metabolism pathway that was determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are colored green colored boxes. Putative metabolites observed in this study are represented with colored circles. Arrows represent direction of enzymatic activity.

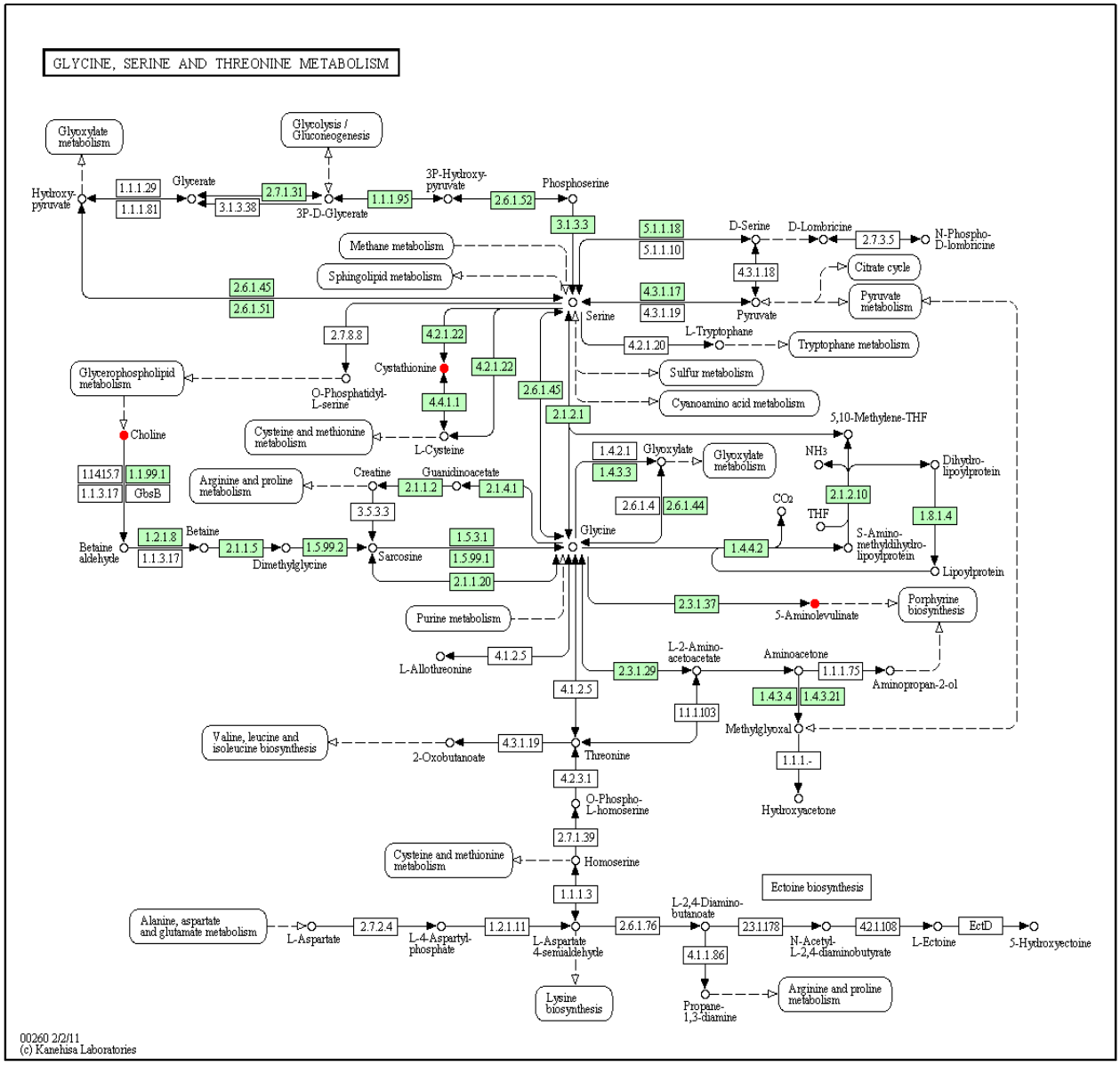


Figure 3. KEGG pathway diagram of glycine, serine and threonine metabolism.

KEGG pathway diagram of the putative metabolites L-cystathionine, choline, and 5-aminolevulinic acid (red dots) in the glycine, serine, and threonine metabolism pathway that were determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

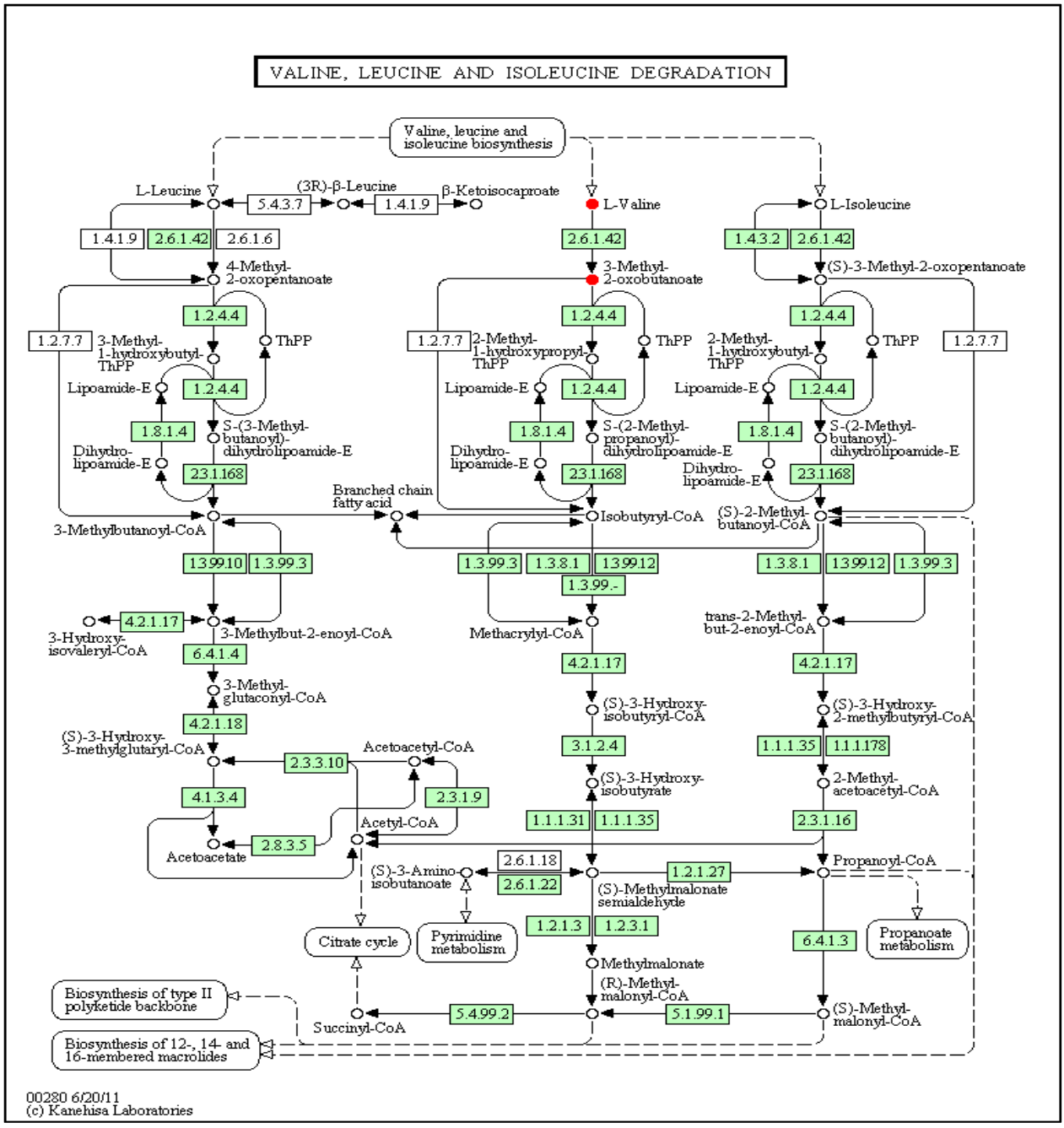


Figure 4. KEGG pathway diagram of valine, leucine and isoleucine degradation.

KEGG pathway diagram of the putatively annotated metabolites valine and 3-methy-2-oxobutanoate (alpha-ketoisovaleric acid) (red dots) in the valine, leucine and isoleucine degradation pathway that were determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

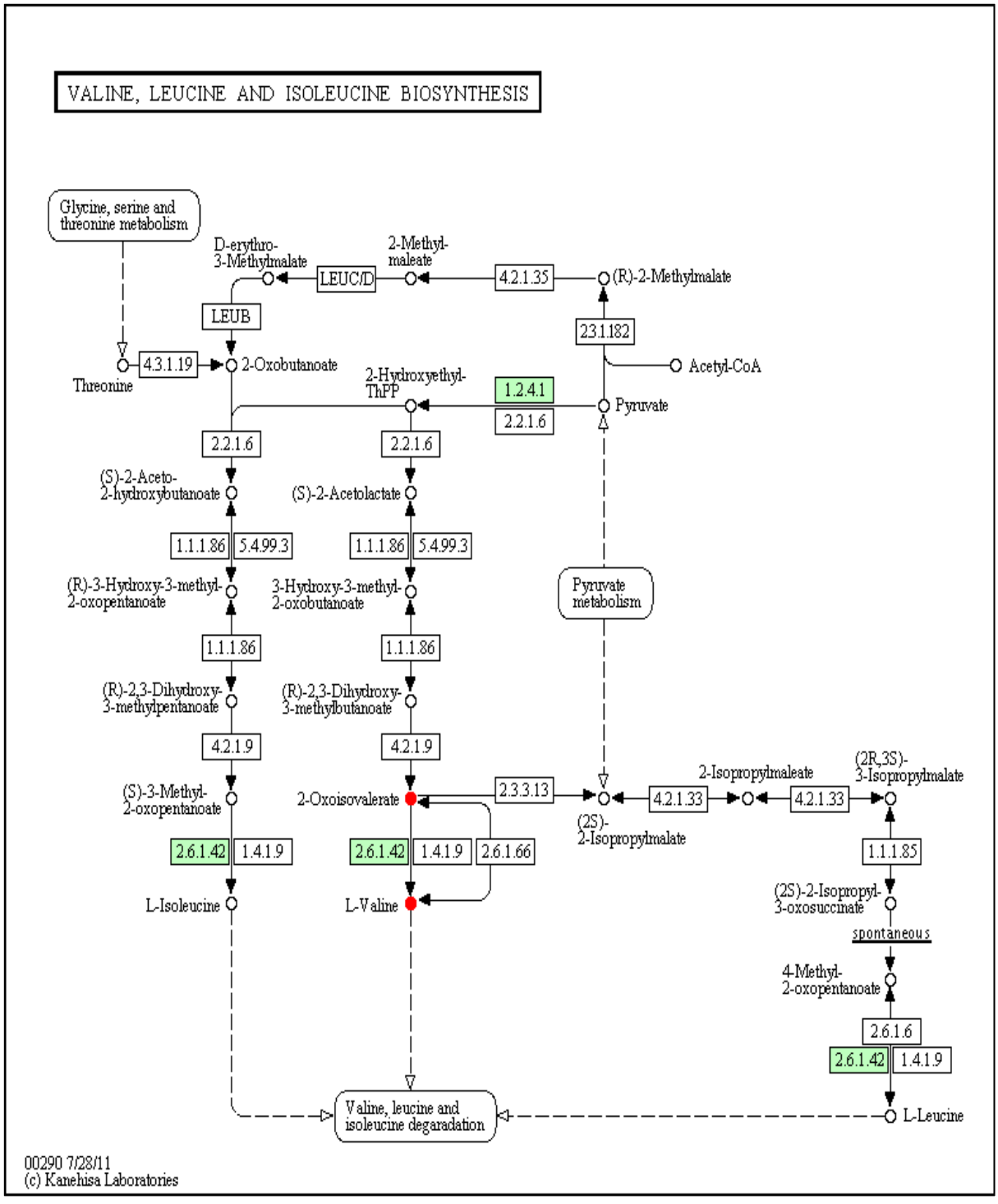


Figure 5. KEGG pathway diagram of valine, leucine and isoleucine biosynthesis.
KEGG pathway diagram of the putatively annotated metabolites valine and 3-methy-2-oxobutanoate (alpha-ketoisovaleric acid) (red dots) in the valine, leucine and isoleucine biosynthesis pathway that were determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

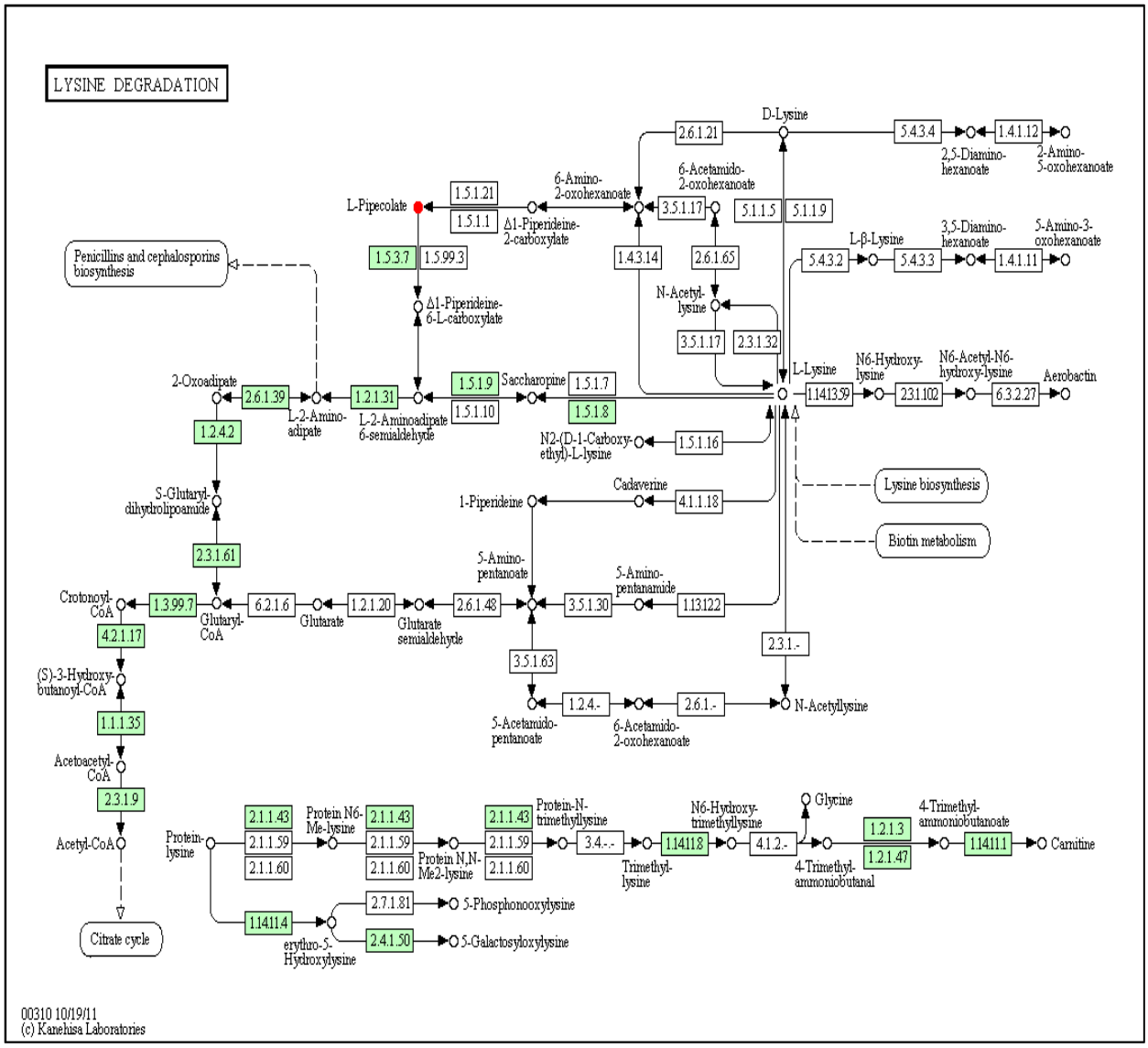


Figure 6. KEGG pathway diagram of lysine degradation.

KEGG pathway diagram of the putatively annotated metabolite L-pipecolate (red dot) in the lysine degradation pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

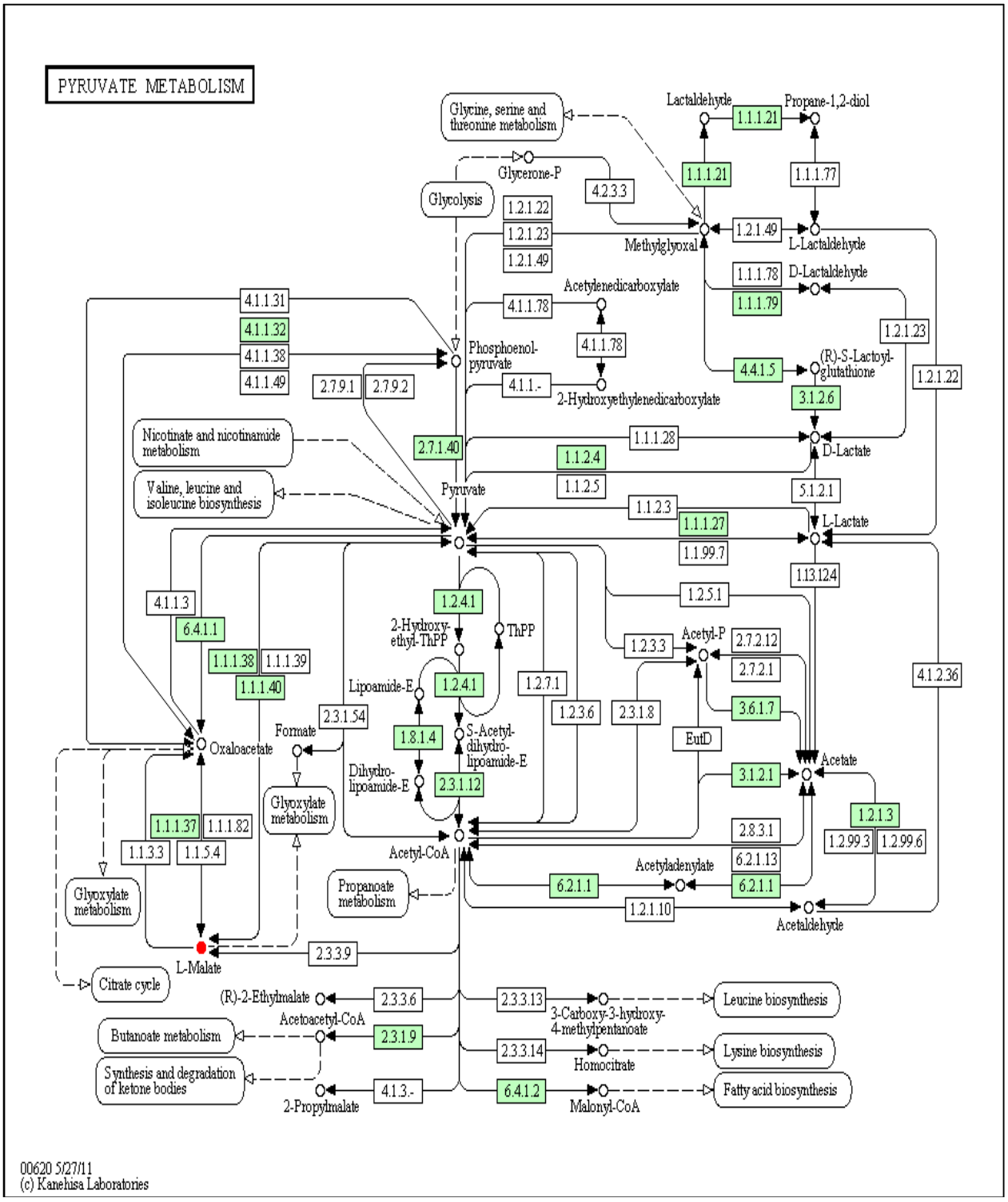


Figure 7. KEGG pathway diagram of pyruvate metabolism.

KEGG pathway diagram of the putatively annotated metabolite L-malate (red dot) in the pyruvate metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

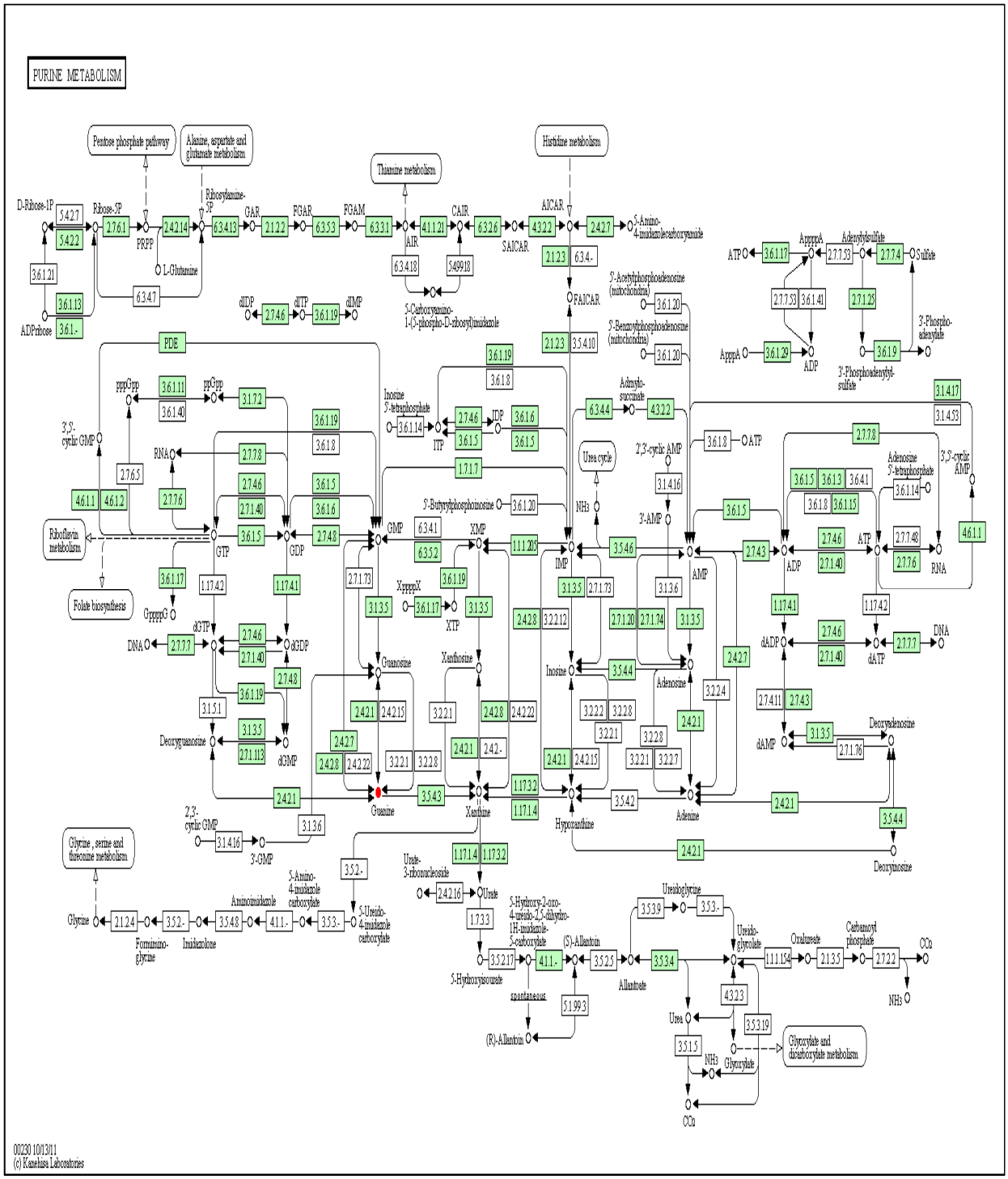


Figure 8. KEGG pathway diagram of purine metabolism.

KEGG pathway diagram of the putatively annotated metabolite guanine (red dot) in the purine metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

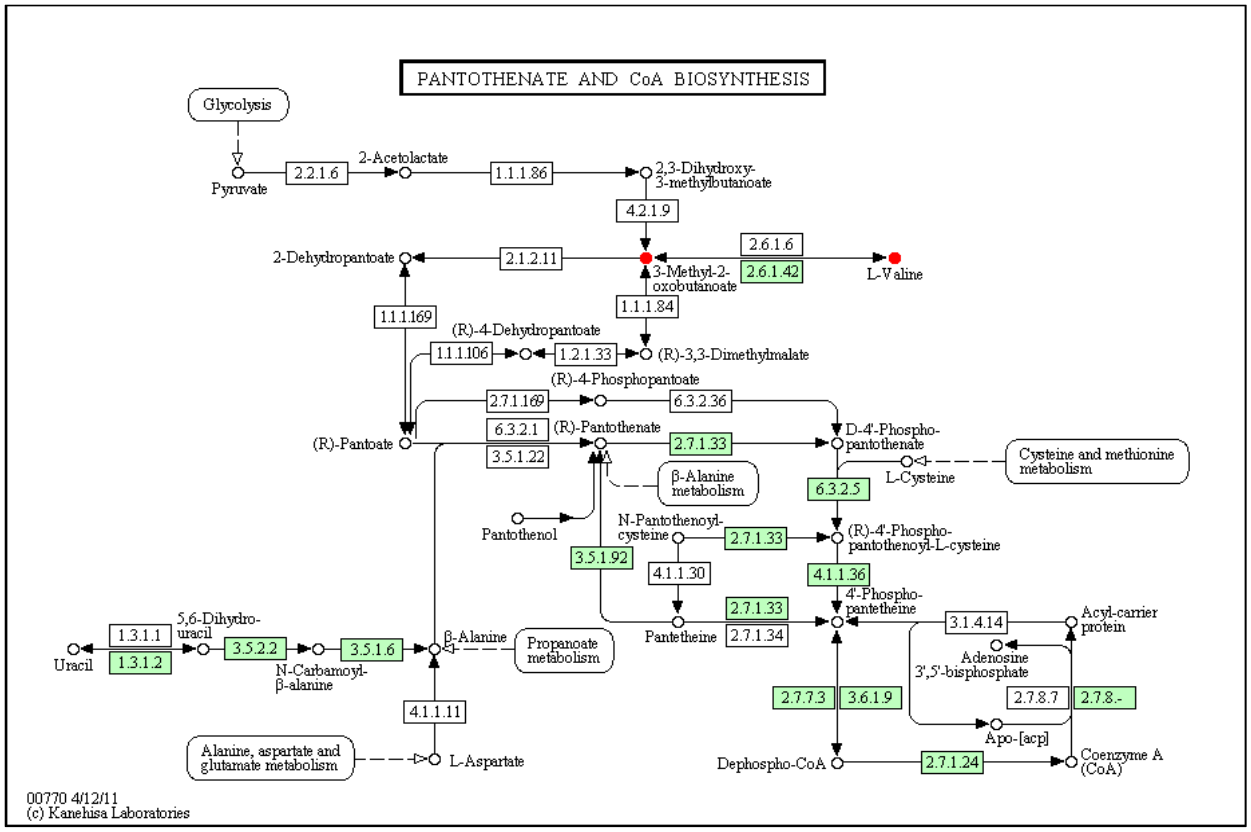


Figure 9. KEGG pathway diagram of pantothenate and CoA biosynthesis.

KEGG pathway diagram of the putatively annotated metabolites L-valine and 3-methyl-2-oxobutanoate (red dots) in the pantothenate and CoA biosynthesis pathway that were determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

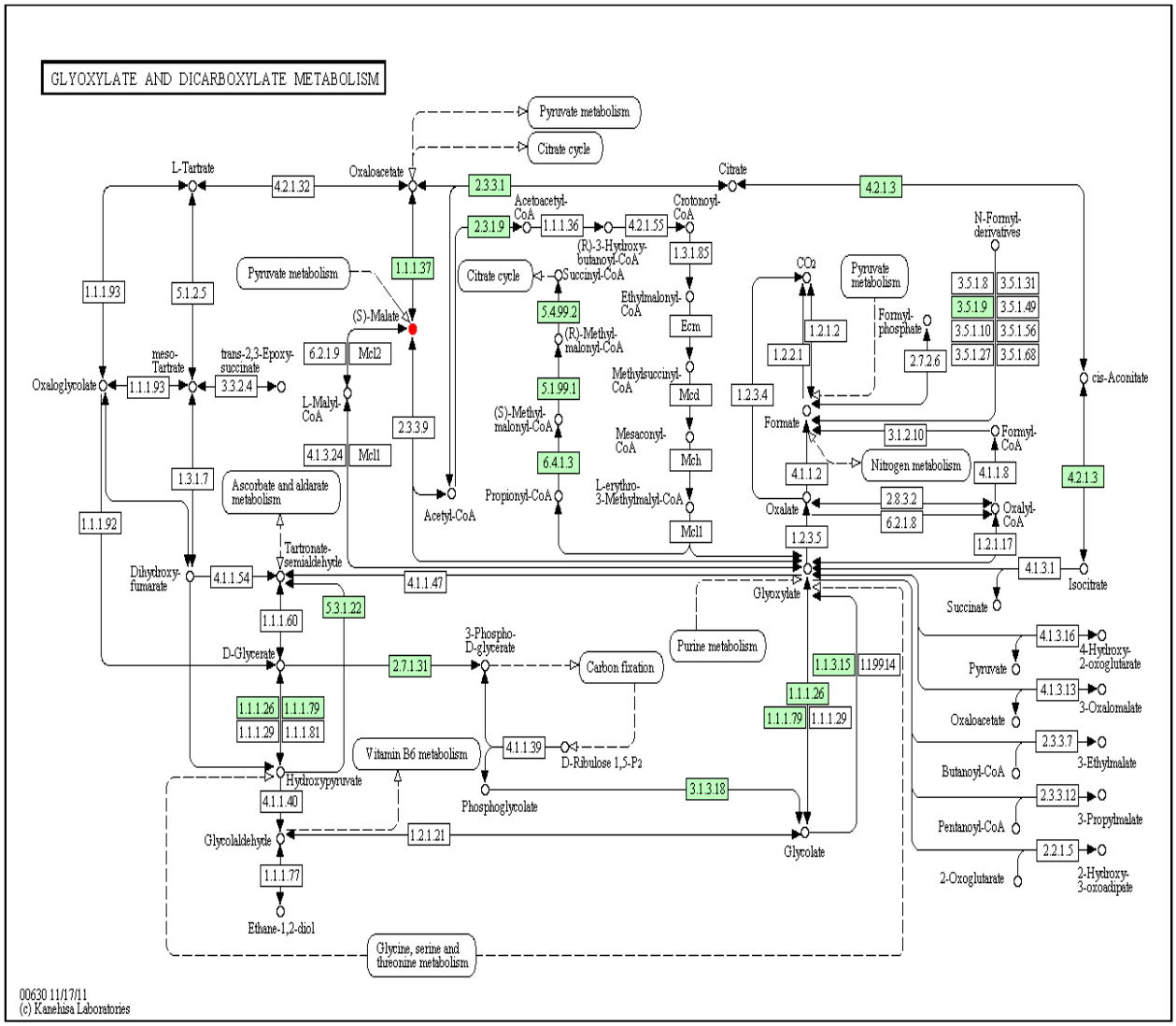


Figure 10. KEGG pathway diagram of glyoxylate and dicarboxylate metabolism.

KEGG pathway diagram of the putatively annotated metabolite S-malate (red dot) in the glyoxylate and dicarboxylate metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

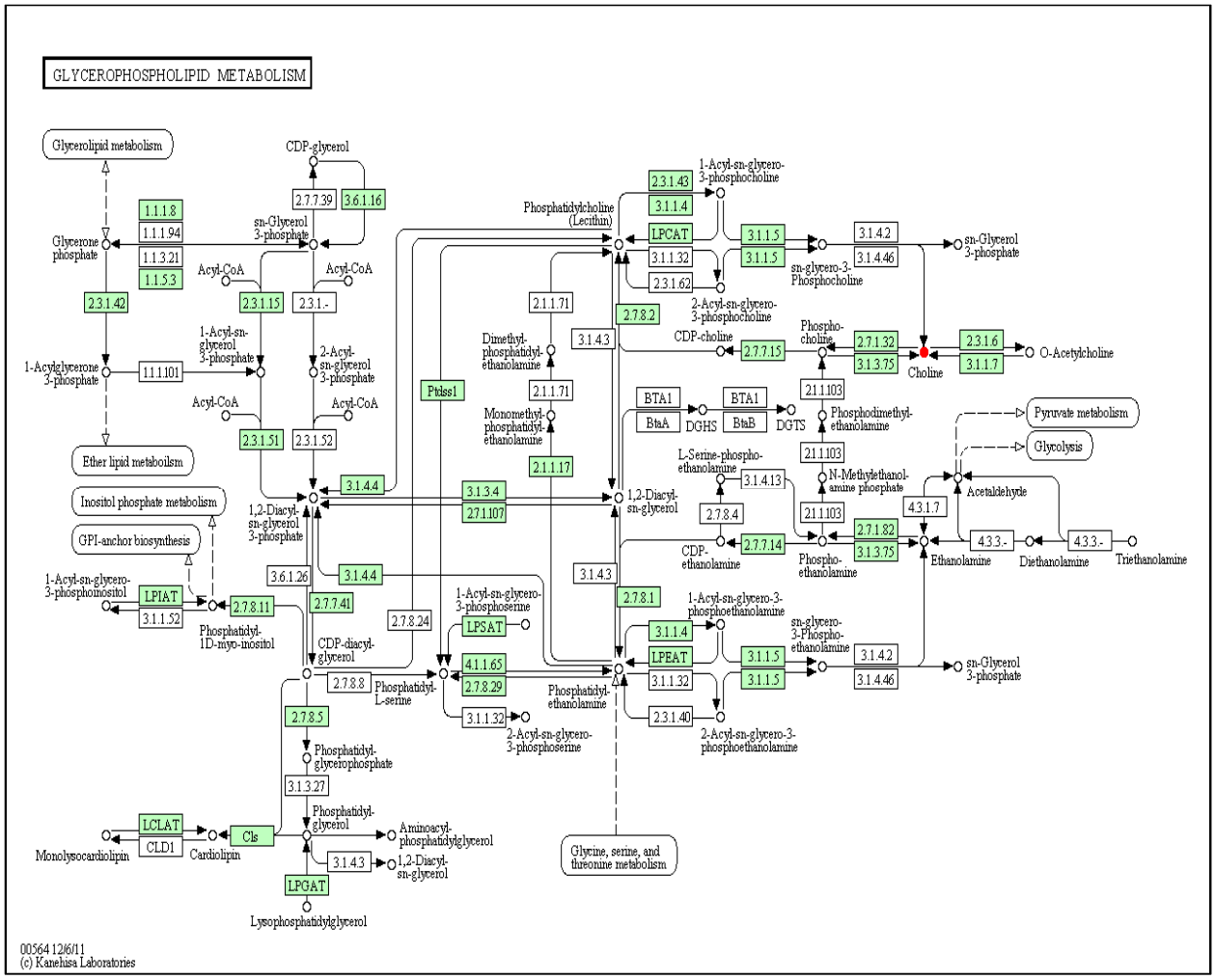


Figure 11. KEGG pathway diagram of glycerophospholipid metabolism.

KEGG pathway diagram of the putatively annotated metabolite choline (red dot) in the glycerophospholipid metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

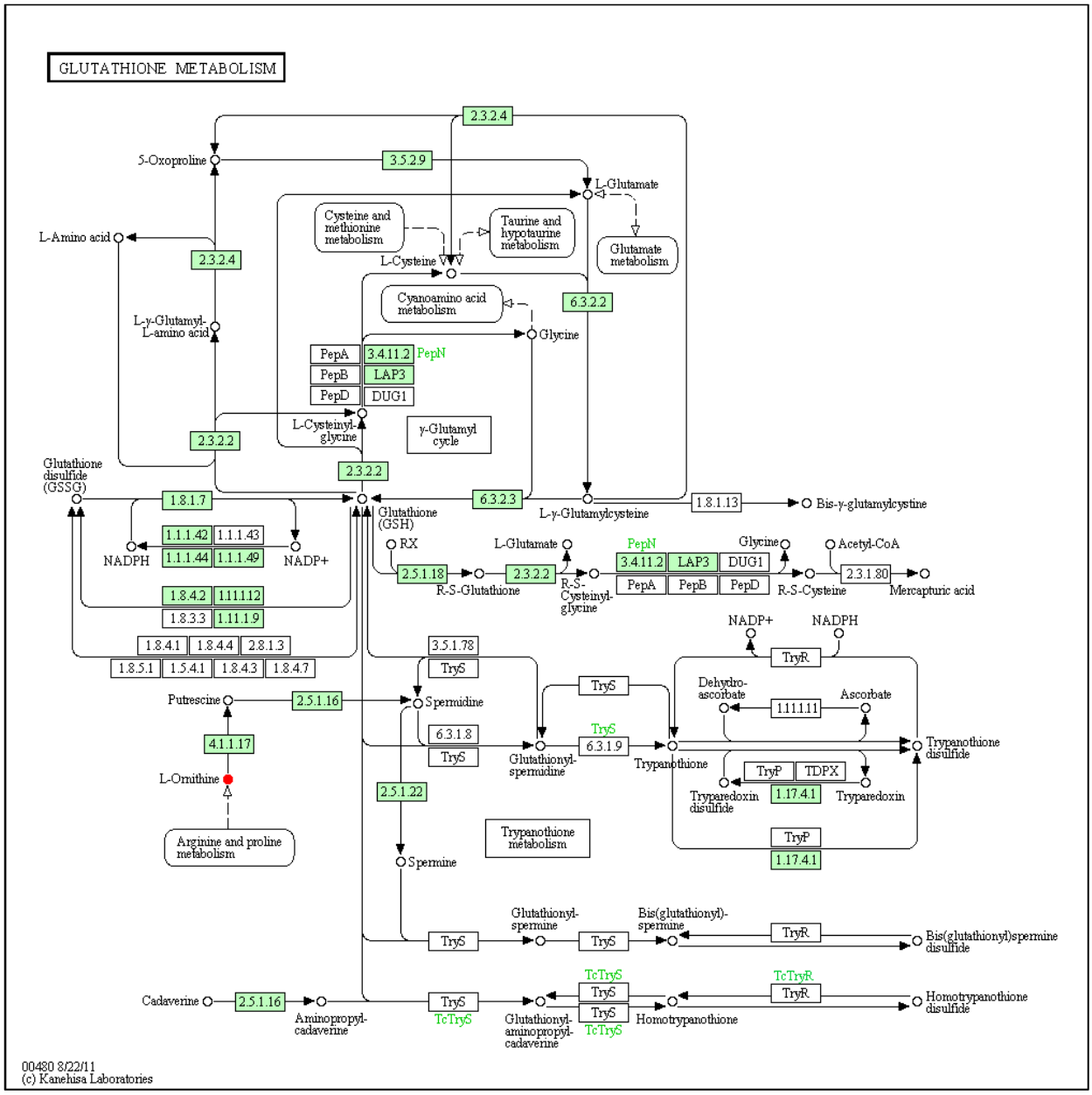


Figure 12. KEGG pathway diagram of glutathione metabolism.

KEGG pathway diagram of the putatively annotated metabolite ornithine (red dot) in the glutathione metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

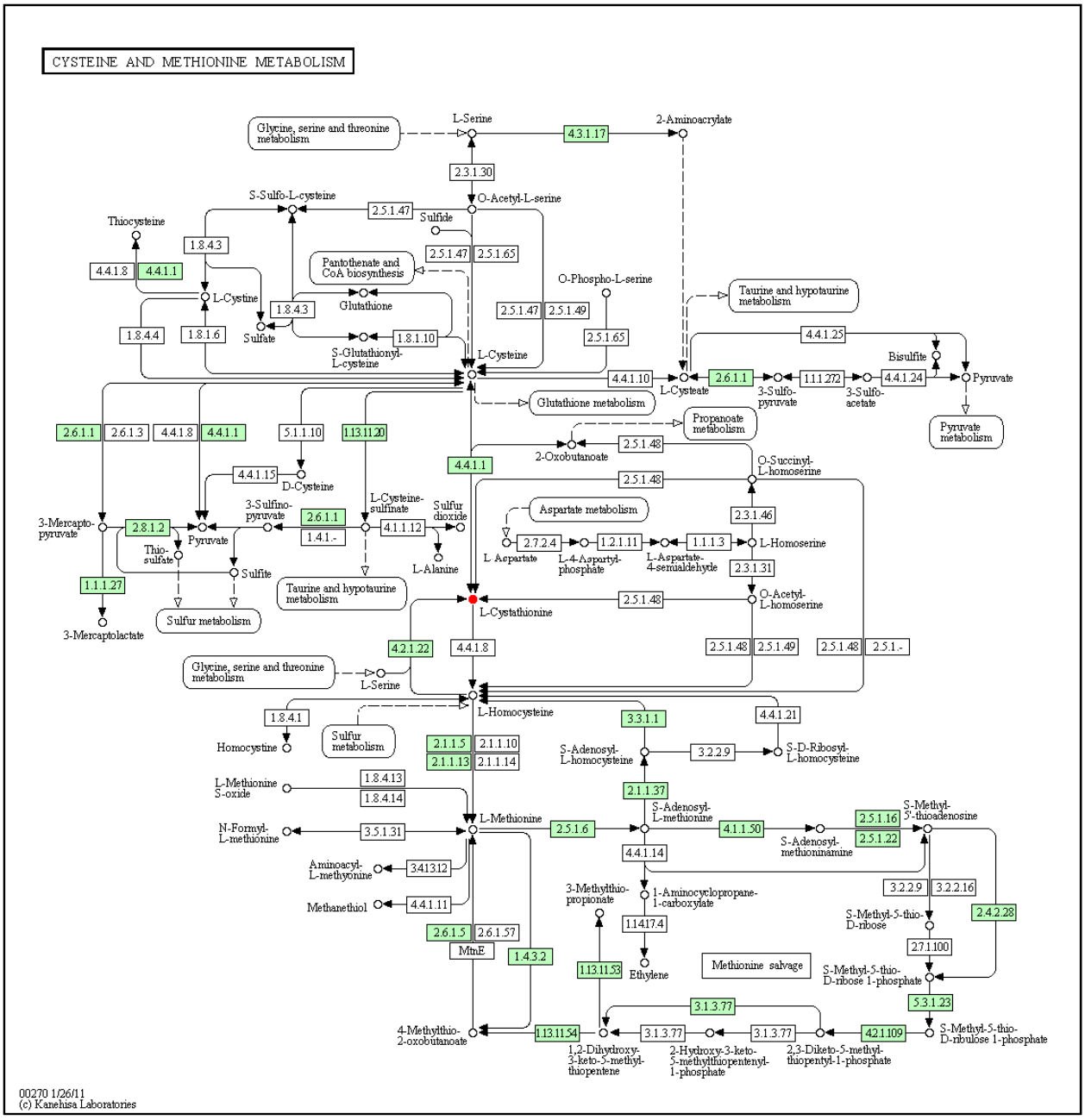


Figure 13. KEGG pathway diagram of cysteine and methionine metabolism.

KEGG pathway diagram of the putatively annotated metabolite L-cystathionine (red dot) in the cysteine and methionine metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. represent direction of enzymatic activity.

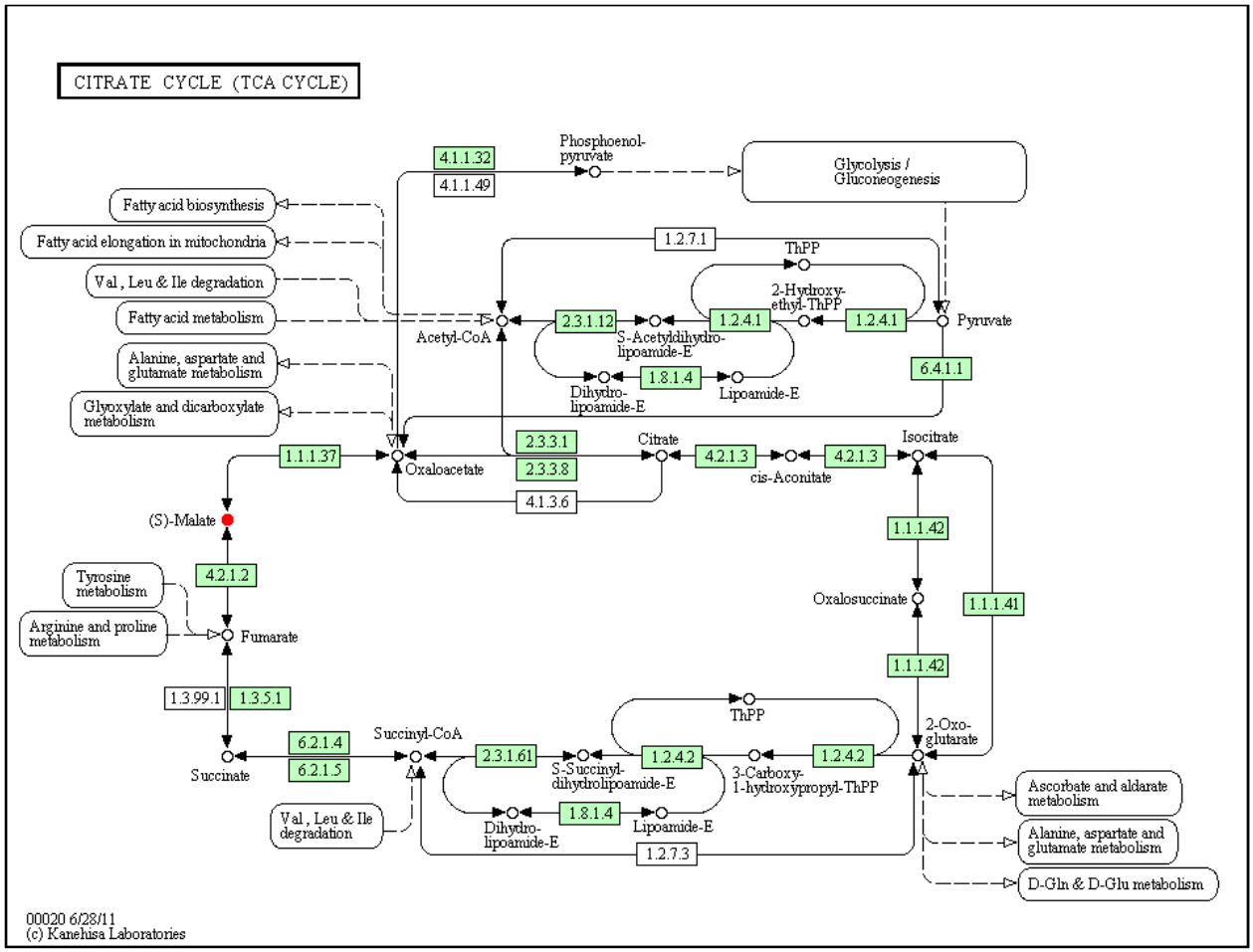


Figure 14. KEGG pathway diagram of citrate cycle (TCA cycle).

KEGG pathway diagram of the putative metabolite malic acid (red dot) in the citrate (TCA) cycle pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

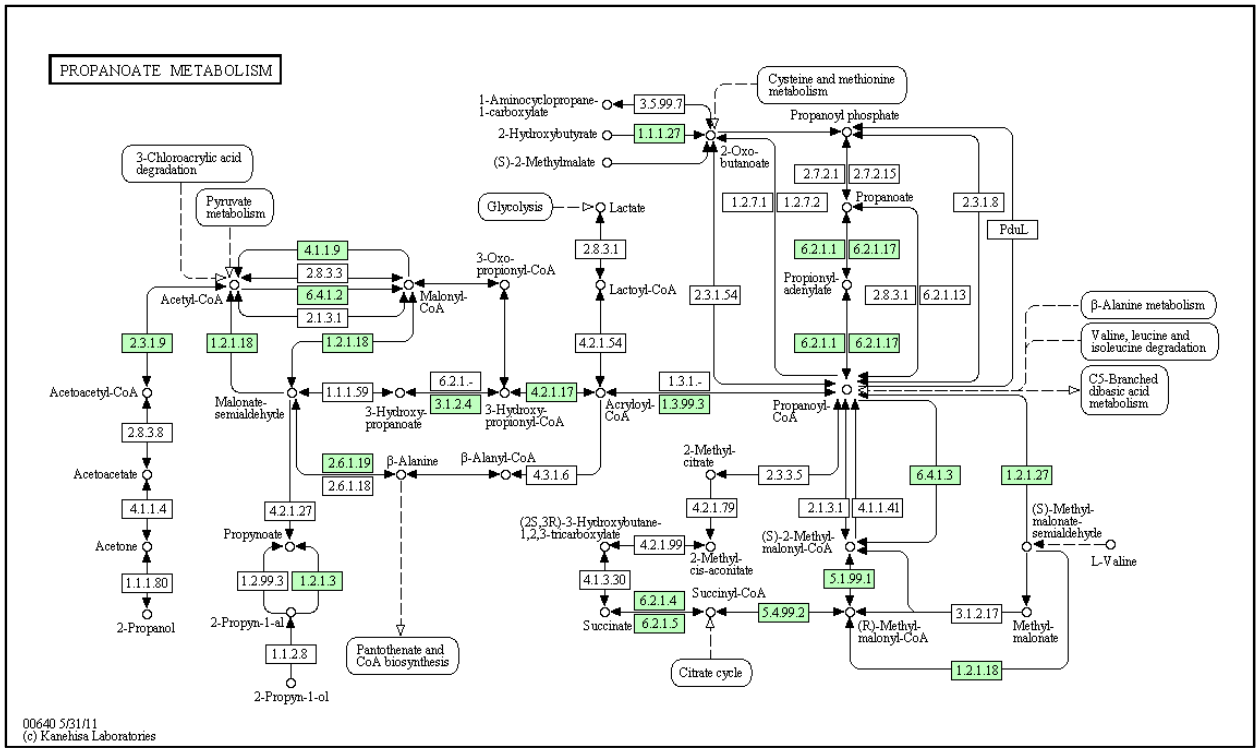


Figure 15. KEGG pathway diagram of propanoate metabolism.
KEGG pathway diagram of the putative metabolite L-valine (red dot) in the propanoate metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

## 5. CONCLUSIONS

As revealed in the Phase I work for this study (Sekowski et al., 2013), several hundred statistically significant differences were observed in the secretomes between exposed and unexposed hESC that indicate MP and MPO have significant impact on hESC metabolism. Putative annotations of those small molecules were identified in Stemina's database of human metabolites. Those statistically significantly enriched metabolites were found to participate in 15 human metabolic pathways. Further literature-based investigation supported that two of those metabolic processes, specifically the arginine and proline metabolism pathway and those processes involved with ROS should be included in subsequent analysis. This Phase II report describes a set of 20 putatively annotated, statistically significant mass features that were both involved with these 15 key metabolic processes and had mass features of sufficient quality for structural confirmation by LC-MS-MS. This set of 20 mass features were downselected to be examined in the Phase III portion of this work (Madren-Whalley, 2013).

LITERATURE CITED

Cezar, G.G.; Quam, J.A.; Smith, A.M.; Guilherme, J.M.R.; Piekarczyk, M.S.; Brown, J.F.; Gag, F.H.; Muotri, A.R. Identification of Small Molecules from Human Embryonic Stem Cells Using Metabolomics. *Stem Cell Dev.* **2007,** *16*(6), 869–892.

Madren-Whalley, J.S.; Palmer, J.S.; Donley, E.L.R.; West, P.; Conard, K.; Sekowski, J.W. *Metabolomic Analysis of the Secretome of Human Embryonic Stem Cells following Methyl Parathion and Methyl Paraoxon Exposure, Phase III: LC-MS-MS Structural Confirmation*; ECBC-TR-1161; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2013; UNCLASSIFIED Report.

Sekowski, J.W.; Bevilacqua, V.L.H.; Palmer, J.A.; Donley, E.L.R.; Burrier, R.E.; Madren-Whalley, J.S. *Metabolomic Analysis of the Secretome of WA09 Human Embryonic Stem Cells following Methyl Parathion and Paraoxon Exposure, Phase I: Initial Nontargeted LC-MS;* ECBC-TR-1177; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2013; UNCLASSIFIED Report.

Blank

# ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| ADMA | asymmetric dimethylarginine |
| APG | Aberdeen Proving Ground |
| CoA | coenzyme A |
| Cys | cysteine |
| ECBC | U.S. Army Edgewood Chemical Biological Center |
| ESI | electrospray ionization |
| FOC | Force Operating Capabilities |
| Gly | glycine |
| hESC | human embryonic stem cell |
| Ile | isoleucine |
| ILIR | in-house laboratory independent research |
| KEGG | Krypto Encyclopedia at Genes and Genomes |
| LC-MS | liquid chromatography followed by mass spectrometry |
| LC-MS-MS | liquid chromatography followed by tandem mass spectrometry |
| Leu | leucine |
| Lys | lysine |
| Met | methionine |
| MP | methyl parathion |
| MPO | methyl paraoxon |
| m/z | mass over charge ratio |
| NOS | nitric oxide synthase |
| Pam | pamphlet |
| ppm | parts per million |
| ROS | reactive oxygen species |
| Ser | serine |
| TCA | trichloroacetic cycle, also known as citrate cycle or Krebs cycle |
| Thr | threonine |
| TIC | toxic industrial chemical |
| TIM | toxic industrial material |
| Val | valine |


EDGEWOOD CHEMICAL BIOLOGICAL CENTER
A U.S. Army RDECOM Laboratory